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# Extracellular Matrix Molecules Involved in Barnacle Shell Mineralization

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## ABSTRACT

***Austromegabalanus psittacus*** is a large ( normally up to 30 cm high) sessile balanomorph barnacle from the coast of Chile and South Peru. Its hard shell is composed of twelve calcareous side plates, six parietes and six radii, joined in the form of a truncated cone opened at the top. Plates rest on a basal disk firmly cemented to the substratum. Although the crystalline microstructure of barnacle's shell has been studied to some extent, its organic composition and the mechanisms governing the biomineralization of such highly ordered nanocomposite have remained obscure. By using X-ray diffraction, infrared spectrometry, SEM and TEM electron microscopy, histochemistry, immuno-histochemistry and –ultrastructure, biochemistry and a crystallization assay, we have studied the cell-shell interactions, the crystalline microstructure of the inorganic moiety and the localization of particular macromolecules, and tested their influence on crystallization.The mineral of the plates and basal disk was calcite showing a (104) preferential orientation. Plates were not solid but porous. While parietes have longitudinal canals (from the base to the apex), radii have transversal canals arranged parallel to the base. These canals are not in the center of the plates but displaced to the outside of the shell delimiting a thinner solid outer lamina and a thicker inner one. The inner lamina consisted of parallel calcified layers separated by organic sheets. These sheets showed autofluorescence and consisted of chitin surrounded by proteoglycans and other minor proteins, which seems to be responsible for the fluorescent behaviour. These organic sheets were also organized as several concentric rings around the canals. The shell matrix obtained after decalcification, which surrounded the crystals, also contained a loose net of such proteoglycans. Mantle epithelial cells covered the entire surface of the inner side of the inner lamina and extend to the plate canals. While isolated chitin did not promote or alter calcite crystallization, the proteoglycan-rich fraction dramatically modified crystal morphology and size. As we have demonstrated in another model of biomineralization, such as the eggshell, hereby we suggest that these structured polyanionic proteoglycan moieties could also be part of the regulatory mechanisms of the barnacle shell mineralization.

## INTRODUCTION

Fabrication of mineralized structures is a widespread phenomenon among living beings [1-3]. Although a great number of these structures are mainly made out of calcium carbonate, they result in the formation of products of unique morphologies and properties. These naturally fabricated bioceramics are composites and are assembled from readily available materials, usually in aqueous solution, at ambient conditions, and to net shape [4]. These particular characteristics reflect the controlling activity of a relatively small repertoire and minute quantities of organic macromolecules which regulate the formation of these structures [5-10]. Some of these macromolecules, obtained from different biomineralization model such as mollusks, crustacea,

echinoderms and avian eggshells, are well characterized, while their role on the control of crystal nucleation, growth and shape is still far from a full understanding [11-13]. While mollusk's shell grows continuously without any spatial restriction, crustacea exoskeleton grows step by step between molting periods. Although being crustacea, showing molting cycle, these Thoraceca barnacles build a quite stable and heavily mineralized wall made out of a series of thick plates that completely surround the animal, which is firmly cemented to the substratum. This makes barnacles an interesting model for studying processes of biomineralization. Since Darwin's pioneering studies [14], there have been several studies on barnacle's shell formation, general structure or microstructure [see 15], but only a few have been concerned with the chemical characterization of the organic matrix of its shell [16]. In this study we therefore examined the shell structure and chemical composition and spatial distribution of its organic matrix.

## MATERIALS AND METHODS

For light microscopy, pieces of shells were polished to obtain thin sheets, mounted on slides and examined with an epifluorescent microscope. For SEM, pieces of shell were polished to obtain thin sheets, decalcified for 15 , 30 and 50 sec with 37 % orthophosphoric acid, dehydrated, coated with gold and observed in a Tesla BS 343 A scanning electron microscope. For TEM, samples were decalcified in 10% formic acid for 48 h at room temperature, dehydrated in a graded acetone series and embedded in Poly Bed 812 (Polysciences Inc., Warrington, PA). Ultrathin sections (70-90 nm) were cut with a Porter Bloom MT2-B ultramicrotome. Bulk ultrastructural visualization of proteoglycans was done by using polyethyleneimine [17]. For precise proteoglycan determination by immunogold localization, section were incubated with one of the primary monoclonal anti-glycosaminoglycan antibodies listed below and then incubated with gold conjugated second antibody (Ted Pella, CA). Observation were made with a Zeiss EM-109 electron microscope.

Antibodies: 2B6 (IgG): recognize dermatan sulfate or chondroitin-4-sulfate after chondroitinase digestion [18]; 5D4 (IgG): recognize a hypersulfated hexasaccharide of keratan sulfate [19].

For organic matrix extraction and *in vitro* crystallization assays, pieces of shell were decalcified using Dowex resin in a rotating glass tube [6] for 3 days at room temperature, dialyzed (Spectra/Por membrane tubing MWCO 3,500) and lyophilized. Organic matrix (soluble and insoluble fractions) obtained was assayed for their crystallization properties. The crystallization experiment were done using a chamber consisting of 85 mm plastic Petri dish having a central hole in its bottom glued to a plastic cylindrical vessel. Inside the chamber, microbridges were filled with 35 µl of 200 mM calcium chloride solution in 200 mM TRIS buffer pH 9. The cylindrical vessel contained 25 mM ammonium carbonate. Control samples contained only calcium chloride solution, while 64 µg/ml of organic matrix from shells were added to the experimental ones. All experiments were carried out inside the petri dish at 20° C for variable periods of time (6-24 h). Precipitation of calcium carbonate results from the diffusion of carbon dioxide vapor into the buffered $CaCl_2$ solution [20]. The crystals were observed in a Tesla BS 343 A scanning electron microscope. The FTIR spectrum was obtained by using a 1% KBr pellet, in the 400- to 4000 $cm^{-1}$ region from crude organic matrix treated with 10% NaOH (standard method for obtaining pure deproteinized chitin) at room temperature compared to standard chitin. For X-ray diffraction analysis, pieces of shell were ground into fine powder, applied to a holder,

and scanned at 6°C/min with a step size of 0.01°, with an operating voltage of 40KV and current of 35 mA in a Siemens D5000 diffractometer.

## RESULTS AND DISCUSSION

Specimens of ***Austromegabalanus psittacus*** are seen in figure 1a. Barnacle's shell is composed of twelve calcitic plates, six parietes and six radii, joined in the form of a truncated cone opened at the top, and resting on a basal disk (figure 1b). While parietes have longitudinal canals (from the base to the apex), radii have transversal canals arranged parallel to the base (figure 1b). These canals are displaced to the outside of the shell delimiting a thinner solid outer lamina and a thicker inner one. The inner lamina consists of parallel calcitic layers separated by organic sheets, which also organize as many concentric rings around the canals (figure 1c and d). These sheets and rings showed autofluorescence (figure 1e and f) and consisted of β chitin (figure 2a). This fluorescence disappeared after mild treatment with NaOH, indicating that some undefined proteins associated to chitin are involved in this phenomenon. Partially decalcified shells show numerous apparently disoriented planar aggregated fusiform calcite crystals located between chitin sheets intermixed with an organic material which eventually collapsed after complete decalcification remaining as a granular sheet (figures 2b and 3a). Chitin sheets showed a parallel fibrillar structure and a less electron-dense granular material between them was observed (figure 3b). A higher magnification of this granular material stained with a cationic dye (polyethyleneimine) showed the occurrence of a net-shape arrangement of proteoglycans (figure 3b, insert). Immunogold studies of this organic matrix showed a positive reaction with anti-keratan sulfate antibody (5D4) around the chitin sheets (figure 3c), while the granular material between the chitin sheets showed a positive reaction with anti-dermatan sulfate antibody (2B6) (figure 3d).

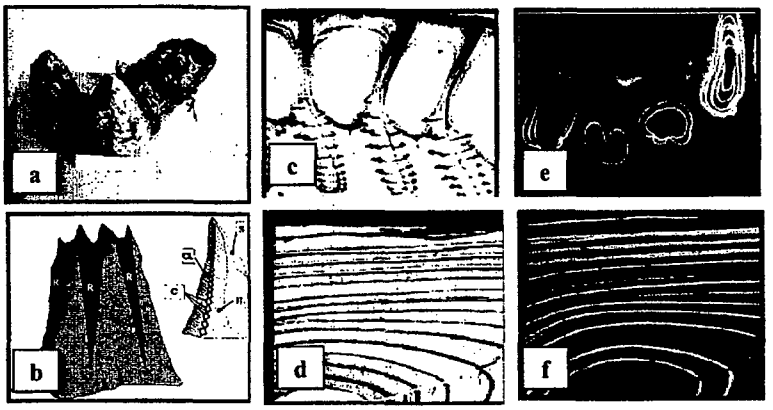


**Figure 1.** a) Live *A. psittacus* specimens from central coast of Chile, bar: 10 cm ; b) Schematic illustration of barnacle's shell wall showing parietes (P) and radii (R), and a longitudinal section of a radius showing canals (C ), outer and inner lamina (OL and IL), and sheath (S); c) and d) Light microscopy of polished transversal section of a paries showing canals and inner lamina respectively, 400X; e) and f) Same as c) and d) but observed under the fluorescence microscopy, showing autofluorescent rings around canals and sheets layering the inner lamina, 400X.

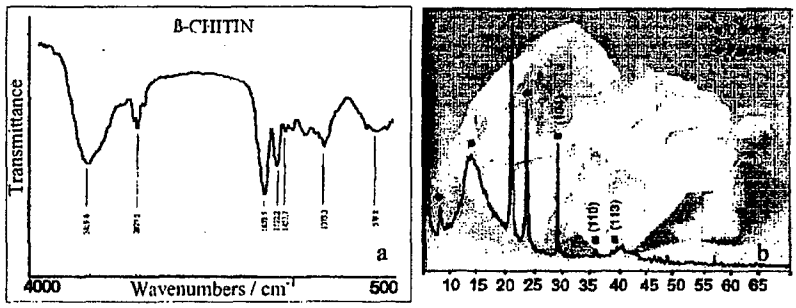


**Figure 2.** a) FITR spectrum of the insoluble organic matrix extracted from barnacle's shell corresponding to β chitin; b) Powder XRD pattern showing preferred orientation of calcite crystals obtained from barnacle's shell

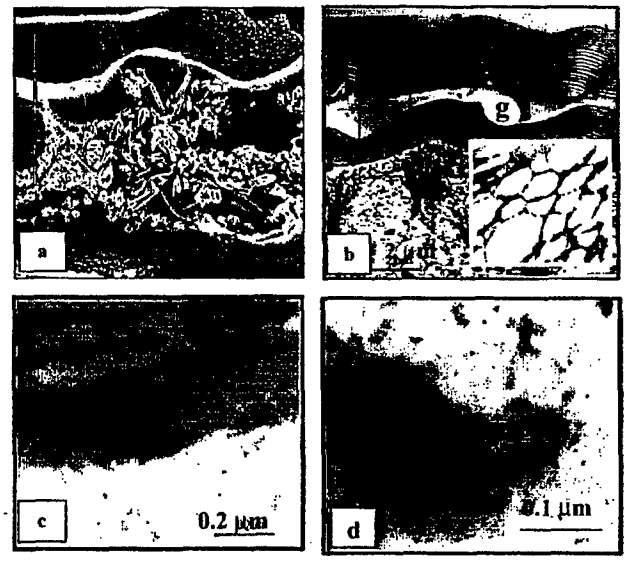


**Figure 3.** a) SEM of partially decalcified shell showing elongated crystals associated to granular organic material between chitin sheets (g); b) TEM of a decalcified shell showing laminated sheets of chitin and a granular material between them (g). Insert: granular material positive to cationic dye indicating protcoglycan occurrence (g); c) immunogold positive reaction with anti-keratan sulfate antibody around chitin fibers; d) immunogold positive reaction with anti-dermatan sulfate antibody on the granular material located between chitin sheets.

When crystallization assays were carried out in the absence of any organic additive, regular calcite crystals showing the {104} faces were obtained (figure 4a). However, when a mixture of soluble and insoluble fractions of shell organic matrix containing chitin, proteoglycans and some proteins was added, a time-depending modification of the calcite morphology was observed. In the first 8 h, crystals seems to nucleate from the chitin fibers, and the corners of the rhombohedra became rough and started to develop curved faces (figure 4b). Between 8 to 20 h, helical aggregations of rounded crystals compacted each other (figure 4c), and after 20 h these rounded crystals appears to be composed of helically ordered planar aggregates of elongated crystals (figure 4d).

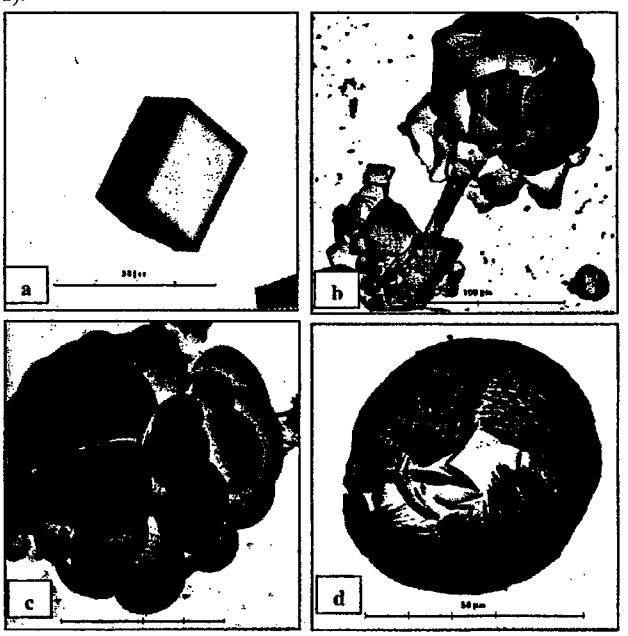


**Figure 4.** SEM micrographs showing the morphology of calcite crystals grown *in vitro* with or without shell organic matrix soluble plus insoluble fractions at different incubations times: a) Control experiment showing the {104} faces; b) incubation with the fractions for 6-8 h; c) incubations with the fractions for 8-20 h; d) incubations with the fractions for 20-24 h.

Although we can not discard the occurrence of undefined proteins in the barnacle's shell, we do show the occurrence and precise localization of particular proteoglycans. The same proteoglycans have been observed in the avian eggshell where a keratan sulfate-rich proteoglycan (mammillan) has a role in the nucleation of the first calcite crystals, and a dermatan sulfate-rich proteoglycan (ovoglycan) regulates the growth and orientation of the later forming crystals [21-22]. The proposed structure of the nacreous organic matrix of molluks includes layers of chitin (with patches of associated anionic-rich proteins) separated by a silk fibroin gel [23]. Barnacle's shell appeared to have a similar general structure, and here we provide evidence of the occurrence of an additional kind of polyanionic, highly hydrophilic and gelatinous molecules, one closely

associated to the chitin fibers (keratan sulfate proteoglycans) and other in the granular material located between them (dermatan sulfate proteoglycans), both localized where the calcite crystals were before decalcification. Foregoing experiments could answer the precise effect of these sulfated molecules in crystal nucleation and growth. However, the extracted barnacle shell organic matrix, containing these sulfated polymers, shows dramatic effects on the morphology of calcite crystals. We suggest that these sulfated macromolecules, because their acidic nature, could be also responsible for controlling the processes of biomineralization of barnacle shell.

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